Appendix A

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Effect of Exogenous and Endogenous Antioxidants on 3-Nitropropionic Acid-Induced In Vivo Oxidative Stress and Striatal Lesions: Insights into Huntington's Disease

*†Michael A. La Fontaine, ‡§James W. Geddes, *Andrea Banks, and *†§D. Allan Butterfield

*Departments of *Chemistry and ‡Anatomy and Neurobiology, †Center of Membrane Sciences, and §Sanders-Brown Center on Aging, University of Kentucky, Lexington, Kentucky; U.S.A.*

Abstract: 3-Nitropropionic acid (3-NP) is an irreversible inhibitor of complex II in the mitochondria. 3-NP toxicity has gained acceptance as an animal model of Huntington's disease (HD). In the present study, we confirmed that rats injected with 3-NP (20 mg/kg, i.p., daily for 4 days) exhibit increased oxidative stress in both striatum and cortical synaptosomes as well as lesions in the striatum. Synaptosomal membrane proteins from rats injected with 3-NP exhibited a decrease in W/S ratio, the relevant electron paramagnetic resonance (EPR) parameter used to determine levels of protein oxidation, and western blot analysis for protein carbonyls revealed direct evidence of increased synaptosomal protein oxidation. Treatment of rats with the brain-accessible free radical spin trap 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO; 30 mg/kg, i.p., daily 2 h before 3-NP injection) or with *N*-acetylcysteine (NAC; 100 mg/kg, i.p., daily 2 h before 3-NP injection), a known glutathione precursor, before 3-NP treatments protects against oxidative damage induced by 3-NP as measured by EPR and western blot analysis for protein carbonyls. Furthermore, both DEPMPO and NAC treatments before 3-NP administration significantly reduce striatal lesion volumes. These data suggest oxidative damage is a prerequisite for striatal lesion formation and that antioxidant treatment may be a useful therapeutic strategy against 3-NP neurotoxicity and perhaps against HD as well. **Key Words:** 3-Nitropropionic acid—Huntington's disease—Glutathione—5-Diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide—*N*-Acetylcysteine—Electron paramagnetic resonance—Oxidative stress.
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Huntington's disease (HD) is an inherited neurodegenerative disorder characterized by progressive choreiform movements, cognitive impairment, and loss of neurons in the striata (Albin et al., 1990; Kremer et al., 1992; Guyot et al., 1997; Nakao and Brundin, 1997). HD is known to be caused by an abnormality in the *IT15* gene (Huntington's Disease Collaborative Research Group, 1993), giving rise to a mutant "huntingtin" protein with an expanded polyglutamine domain. Although mutant huntingtin has been reported to associate with glyceraldehyde 3-phosphate dehydrogenase and possibly to impair activity of aconitase (Burke et al., 1996; Tabrizi et al., 1999, 2000), the underlying mechanisms of selective striatal damage are unknown. Recently, the activation of excitatory amino acid receptors has been implicated in HD (Albin and Greenamyre, 1992; Beal, 1992). In addition, several mitochondrial toxins, when injected in rats, have been found to cause behavioral alterations and striatal lesions similar to the neurochemical and anatomical changes associated with HD (Beal et al., 1991, 1993*a*; Brouillet et al., 1993; Koutouzis et al., 1994; Schulz et al., 1995). One such toxin that is gaining prominence for use in animal models of HD is the mitochondrial toxin 3-nitropropionic acid (3-NP) (Beal et al., 1993*b*; Brouillet et al., 1993; Guyot et al., 1997). 3-NP is an irreversible inhibitor of the electron transport enzyme succinate dehydrogenase (Beal, 1992; Ludolph et al., 1992; Brouillet et al., 1993; Palfi et al., 1996).

Oxidative stress has been suggested to play a role in 3-NP toxicity; however, the processes behind oxidative damage are not fully understood (Schulz et al., 1996). NMDA receptors can be activated by 3-NP, leading to generation of superoxide radicals (Lafon-Cazal et al., 1993) and allowing calcium influx into the cell, both of which can further exacerbate oxidative damage (Wullner et al., 1994). Other evidence has also supported the theory of free radical involvement with excitotoxicity (Coyle and Puttfarcken, 1993), and our laboratory has directly measured increased protein oxidation on admin-

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Address correspondence and reprint requests to Prof. D. A. Butterfield at Department of Chemistry and Center of Membrane Sciences, University of Kentucky, Lexington, KY 40506-0055, U.S.A. E-mail: dabcns@pop.uky.edu

Abbreviations used: DEPMPO, 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide; EPR, electron paramagnetic resonance; HD, Huntington's disease; MAL-6, 4-maleimido-2,2,6,6-tetramethylpiperidine-*N*-oxyl; NAC, *N*-acetylcysteine; 3-NP, 3-nitropropionic acid.

istration of 3-NP to rats and showed that oxidative stress preceded the formation of striatal lesions (La Fontaine et al., 2000).

In the present study, we examined whether the brain-accessible spin trap 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO) can attenuate oxidative stress caused by 3-NP toxicity as measured by electron paramagnetic resonance (EPR) and protein carbonyl analyses. The ability of DEPMPO to protect against 3-NP-induced striatal lesions also was examined. In addition, the importance of endogenous antioxidants in protection against 3-NP-induced oxidative stress was studied. We reasoned that elevation of levels of the endogenous antioxidant glutathione should be protective. Accordingly, the effect of increasing glutathione levels using the glutathione precursor *N*-acetylcysteine (NAC) on 3-NP toxicity was investigated. NAC, when injected in rodents, has been found to increase brain glutathione levels significantly (Testa et al., 1998; Pocernich et al., 2000). Taken together, these studies may offer insight into the role of oxidative stress in 3-NP-induced in vivo toxicity and lesion formation and possibly the neurochemistry and neuropathology of HD.

MATERIALS AND METHODS

Chemicals

3-NP was obtained from Aldrich Chemical. Ultrapure sucrose, 4-maleimido-2,2,6,6-tetramethylpiperidine-*N*-oxyl (MAL-6), NAC, and anti-rabbit IgG antibody were obtained from Sigma Chemical Co. DEPMPO was obtained from Oxis International. The protease inhibitors aprotinin, leupeptin, and pepstatin A were obtained from Calbiochem. The OxyBlot oxidized protein detection kit was obtained from Oncor. All remaining chemicals were obtained from Sigma in the highest possible purity.

Animals

All animal protocols have been approved by the University of Kentucky Animal Care and Use Committee. Male rats were purchased from Harlan and housed in the Sanders-Brown Center on Aging Animal Care Facility. The Sprague–Dawley rats were exposed to 12-h light/12-h dark conditions and were fed Purina Rodent Laboratory Chow with no restrictions to feed or water. At 4 months of age, rats treated with 3-NP were injected intraperitoneally daily for 4 days with 3-NP dissolved in physiologic saline at a dose of 20 mg/kg, pH 7.4. Control animals received corresponding injections of physiologic saline. DEPMPO-treated animals were injected intraperitoneally daily with 30 mg/kg DEPMPO dissolved in physiologic saline 2 h before 3-NP treatment. NAC-treated animals were injected intraperitoneally daily with 100 mg/kg NAC dissolved in physiologic saline 2 h before 3-NP treatment. Animals were anesthetized with sodium pentobarbital and decapitated 24 h after the final injection.

Synaptosome preparation

Synaptosomes were purified as previously described (Butterfield et al., 1994; Hensley et al., 1994). The homogenate was respun at 20,000 *g* at 4°C for 10 min. The resulting pellet was suspended in ~1 ml of isolation buffer and layered on a discontinuous sucrose gradient [10 ml of 1.18 *M* sucrose (pH 8.5)/10 ml of 1.0 *M* sucrose (pH 7.4)/10 ml of 0.85 *M* sucrose (pH 7.4), each containing 2 m*M* EDTA, 2 m*M* EGTA, and 10 m*M* HEPES]. The samples were then spun at 82,500 *g* for 2 h at 4°C in a Beckman swinging-bucket rotor. Synaptosomes were collected from the 1.18/1.0 *M* sucrose interface and resuspended in 20 ml of lysing buffer (10 m*M* HEPES, 2 m*M* EDTA, and 2 m*M* EGTA, pH 7.4). The samples were then spun at 32,000 *g* at 4°C for 10 min. The pellet was resuspended in lysing buffer and spun twice more. After the third spin, the protein concentration was determined by the method of Lowry et al. (1951).

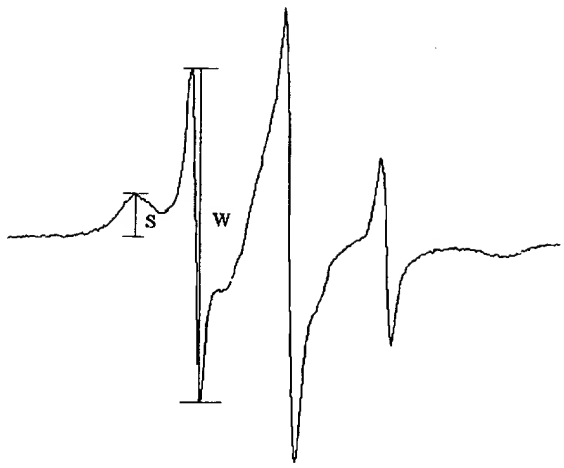

FIG. 1. A typical EPR spectrum of MAL-6-labeled synaptosomal membrane proteins depicts the W and S components of the low-field resonance line.

Spin labeling

Each sample was separated into 4-mg aliquots. Spin labeling of synaptosomal membrane proteins was done as previously described (Umhauer et al., 1992; Hensley et al., 1994). Lysed synaptosomal membranes were labeled with 20 μg of MAL-6/mg of protein. After an 18-h incubation at 4°C, samples were washed six times with lysing buffer to remove excess spin label. Each 4-mg pellet was resuspended in a 1 ml of lysing buffer. EPR spectra were acquired on a Bruker model EMX EPR spectrometer operating at an incident microwave power of 16 mW, a modulation amplitude of 0.4 G, a time constant of 1.28 ms, and a conversion time of 10 ms.

The W/S ratio (Fig. 1) of EPR spectra from MAL-6-labeled membrane proteins has been extensively studied in both brain synaptosomal membranes and erythrocyte membranes (Hall et al., 1995*a*; Hensley et al., 1995; Howard et al., 1996; Butterfield et al., 1997). Increased steric hindrance of the protein-bound spin label will cause a decrease in the W/S ratio. This can be caused by various changes in the environment of the spin label, including altered protein conformation, a decrease in segmental motion in spin labeled proteins, and/or changes in the interactions between proteins. Several oxidative conditions used in our laboratory, including Fenton chemistry to produce hydroxyl radicals (Hensley et al., 1994), hyperoxia (Howard et al., 1996), ischemia–reperfusion (Hall et al., 1995*a–c*), accelerated aging (Butterfield et al., 1997), β-amyloid-derived free radicals (Hensley et al., 1995; Butterfield, 1997; Butterfield et al., 1997; Subramaniam et al., 1998), lipid peroxidation products (Subramaniam et al., 1997), and menadione (Trad and Butterfield, 1994), have shown that an increase in protein oxidation is associated with a decrease in the W/S ratio.

Protein carbonyl measurements

Protein carbonyls are an index of protein oxidation (Butterfield and Stadtman, 1997). To determine the level of protein oxidation, an oxidized protein detection kit based on immunochemical detection of protein carbonyl groups derivatized with 2,4-dinitrophenylhydrazine was used. Synaptosomal membrane proteins were isolated as above and treated with 20 m*M* 2,4-dinitrophenylhydrazine in 10% trifluoroacetic acid and Derivatization-Control solution and incubated for 20 min. Derivatization was neutralized with OxyBlot neutralization solution (2 *M* Tris/30% glycerol) and 19% 2-mercaptoethanol.

Polyacrylamide gel electrophoresis was performed in mini-slabs (0.75 × 60 × 70 mm, 12% acrylamide) according to the method of Laemmli (1970). Following electrophoresis, proteins were transferred to nitrocellulose paper (pore size, 0.45 μm) according to the procedure adapted from Glenney (1986). Tris-glycine and 20% methanol at a pH of 8.5 was used as the transfer buffer. Following transfer, nitrocellulose paper was blocked in 3% bovine serum albumin (in phosphate-buffered saline with 0.01% sodium azide and 0.2% Tween-20) for 1 h at room temperature. Membranes were washed three times with washing buffer (1% NaCl, 2% phosphate-buffered saline, 0.01% sodium azide, and 0.1% Tween-20). To the membranes, rabbit anti-2,4-dinitrophenol antibody (1:150 dilution in 90% washing buffer/10% blocking buffer) was added and incubated at room temperature for 1 h under mild shaking. Following incubation, membranes were washed three times with washing buffer. Anti-rabbit IgG (1:15,000 dilution in blocking buffer) was added to the membranes and incubated at room temperature for 1 h with mild shaking. Following incubation, membranes were washed three times and then developed using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium solution (1 SigmaFast tablet per 10 ml of deionized water).

Western blots were analyzed using computer-assisted imaging software (MCID/M4; Imaging Research, St. Catharine's, Ontario, Canada).

Lesion volume analysis

Lesion volumes in the striatum were analyzed by freezing the intact brain on dry ice on removal from decapitated rats. Twenty-micrometer slices with an intersectional distance of 180 μm were mounted on microscope slides and stained using purple cresol tissue stain. The lesion volume of individual slices was analyzed using NIH Image version 6.1 for the Macintosh on images collected using a 2× ocular. Lesion volume was obtained by multiplying the total lesion areas times the intersectional distances.

Statistical analysis

One-way ANOVA was used for comparison of the means. Student's *t* test was used where applicable. Results are expressed as mean ± SD data.

RESULTS

The W/S ratios of MAL-6-labeled synaptosomal membrane proteins isolated from rats treated with 3-NP were significantly decreased in both striatum and cortex when compared with control animals injected with saline vehicle (Fig. 2). Confirming the EPR results, protein carbonyl levels in synaptosomes isolated from rats treated with 3-NP were significantly higher than control levels (Fig. 3). Animals treated with the free radical spin trap DEPMPO before 3-NP administration exhibited a significant decrease in oxidative stress. The W/S ratios of MAL-6-labeled synaptosomal membranes isolated from DEPMPO-treated animals were significantly higher than those of animals treated with 3-NP only (Fig. 2). Protein carbonyl levels were also significantly decreased in synaptosomes isolated from animals treated with DEPMPO compared with animals treated with 3-NP only (Fig. 3). Animals treated with NAC exhibited a similar decrease in oxidative stress, by both EPR analysis (Fig. 2) and protein carbonyl analysis (Fig. 3). Treatment with either DEPMPO or NAC reduced striatal lesion volumes compared with animals treated with 3-NP only (Fig. 4).

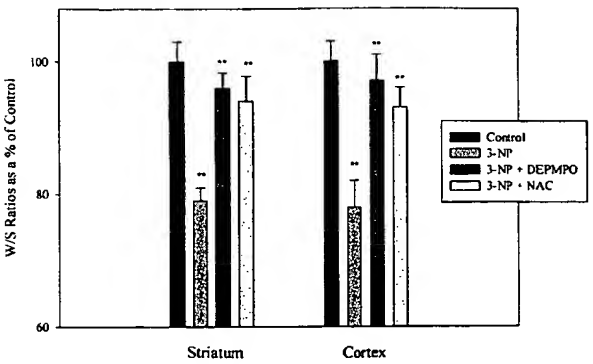

FIG. 2. Effect of DEPMPO and increased glutathione levels (via NAC) on the physical state of synaptosomal membrane proteins assessed by EPR in conjunction with the protein-specific spin label MAL-6. Synaptosomal membrane proteins were isolated from the striatum and cortex of rats treated with 3-NP, 3-NP and DEPMPO, 3-NP and NAC, and saline (control). Synaptosomes were labeled with MAL-6, and EPR spectra were analyzed. Significant decreases in W/S ratios, consistent with protein oxidation (see text), were observed in both brain regions in animals treated with 3-NP compared with control (striatum, control n = 10, 3-NP n = 9, $p < 0.01$; cortex, control n = 11, 3-NP n = 10, $p < 0.01$). W/S ratios of MAL-6-labeled synaptosomes isolated from animals coinjected with DEPMPO were significantly higher in both brain regions than W/S ratios of MAL-6-labeled synaptosomes isolated from animals treated with 3-NP only (striatum, 3-NP + DEPMPO n = 6, $p < 0.01$; cortex, 3-NP + DEPMPO n = 6, $p < 0.01$). W/S ratios of MAL-6-labeled synaptosomes isolated from animals coinjected with NAC were also significantly higher compared with animals treated with 3-NP only (striatum, 3-NP + NAC n = 7, $p < 0.01$; cortex, 3-NP + NAC n = 6, $p < 0.01$). $^{**}p < 0.01$.

Figure 5 shows a cross-section from striatum of 3-NP- and 3-NP plus DEPMPO-treated animals.

Throughout the course of this study, three animals treated with 3-NP only died before the time of harvesting. Therefore, brain tissue analyses from these animals were not conducted. All other test groups exhibited a zero mortality.

DISCUSSION

The effect of 3-NP administration on rodent behavior, physical dexterity, and neuropathology has been studied by several groups; 3-NP is unique among toxin models of HD in that it can be used to mimic the two-stage progression of HD by leading to both hyperactivity and

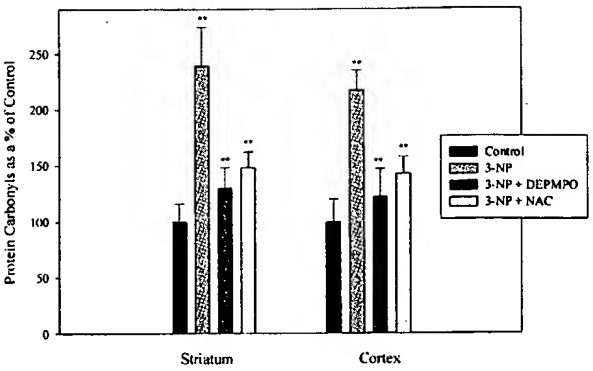

FIG. 3. Effect of DEPMPO and increased glutathione levels (via NAC) on protein oxidation of synaptosomal proteins assessed by levels of protein carbonyls after 3-NP treatment. Synaptosomes were isolated from animals treated with 3-NP, 3-NP and DEPMPO, 3-NP and NAC, and saline (control) and analyzed for protein carbonyl content. A significant increase in levels of protein carbonyls was observed in both brain regions in animals treated with 3-NP compared with control (striatum, control n = 9, 3-NP n = 9, $p < 0.01$; cortex, control n = 10, 3-NP n = 10, $p < 0.01$). Protein carbonyl levels of synaptosomes isolated from animals coinjected with DEPMPO were significantly lower in both brain regions than protein carbonyls in synaptosomes isolated from animals treated with 3-NP only (striatum, 3-NP + DEPMPO n = 6, $p < 0.01$; cortex, 3-NP + DEPMPO n = 6, $p < 0.01$). Protein carbonyl levels of synaptosomes isolated from animals coinjected with NAC were also significantly lower compared with animals treated with 3-NP only (striatum, 3-NP + NAC n = 6, $p < 0.01$; cortex, 3-NP + NAC n = 6, $p < 0.01$). $^{**}p < 0.01$.

hypoactivity (Borlongan et al., 1997). A correlation between severity of 3-NP-induced striatal lesions and motor deficits, including bradykinesia, gait length, and gait velocity, was shown by Guyot et al. (1997) along with regional selectivity similar to that seen in HD. Tsai et al. (1997) demonstrated a dose-dependent decrease in glutamine synthetase activity with 3-NP injections. These researchers also showed an age-dependent increase in susceptibility toward 3-NP toxicity. The decrease of glutamine synthetase activity coupled with the age-dependent susceptibility suggests an oxidative mechanism underlying 3-NP toxicity (Hensley et al., 1995; Aksenov et al., 1997; Butterfield et al., 1997). In addition, the deficiencies in behavior and motor control are reminiscent of the loss of motor skills associated with increased brain protein oxidation (Forster et al., 1996).

Considerable evidence exists to support an oxidative process underlying 3-NP toxicity. Beal et al. (1995) showed an attenuation of 3-NP toxicity in copper/zinc superoxide dismutase-overexpressing transgenic mice. The hydroxyl radical products 2,3-dihydroxybenzoic acid and 2,5-dihydroxybenzoic acid were detected also by these researchers as indirect evidence of hydroxyl radical formation. Pang and Geddes (1997) showed that one mechanism of cell death in 3-NP toxicity was excitotoxic necrosis. Consistent with these researchers, our laboratory has shown an increase in protein oxidation in striatal synaptosomal membrane proteins of rats exposed to 3-NP (La Fontaine et al., 2000).

Despite the evidence of oxidative stress in 3-NP toxicity, the role of reactive oxygen species is not clear. Given the oxidative nature of 3-NP toxicity, we reasoned that the brain-accessible spin trap DEPMPO would modulate protein oxidation in this model of HD. DEPMPO is a phosphorylated analogue of 5,5-dimethyl-1-pyrroline *N*-oxide that exhibits greater stability and can be used in spin-trapping studies to discriminate specific radical species (Clement et al., 1998). We found a significant decrease in levels of protein carbonyls due to 3-NP toxicity in rats cotreated with DEPMPO. Furthermore, we found a significant increase in W/S ratios of the DEPMPO-treated rats compared with those that were not cotreated with DEPMPO. In both EPR experiments and protein carbonyl level measurements, DEPMPO protection gave mean values of the respective parameters that were close to values for control animals. The commonly used spin trap α-phenyl-*N*-*tert*-butyl nitrone and analogues were not considered for this study owing to their apparent exacerbation of 3-NP toxicity (Schulz et al., 1996; Nakao and Brundin, 1997).

Evidence of decreased glutathione levels in 3-NP-treated animals and attenuation of 3-NP toxicity in copper/zinc superoxide dismutase transgenic mice (Beal et al., 1995) suggest endogenous antioxidants may play a role in protecting against 3-NP-induced oxidation. Our laboratory reported that increased glutathione levels resulting from NAC injections protect rat synaptosomes from oxidation caused by hydroxyl radicals (Pocernich et al., 2000). In support of the hypothesis that glutathione performs an antioxidant role in 3-NP toxicity, we report a significant decrease in synaptosomal protein oxidation

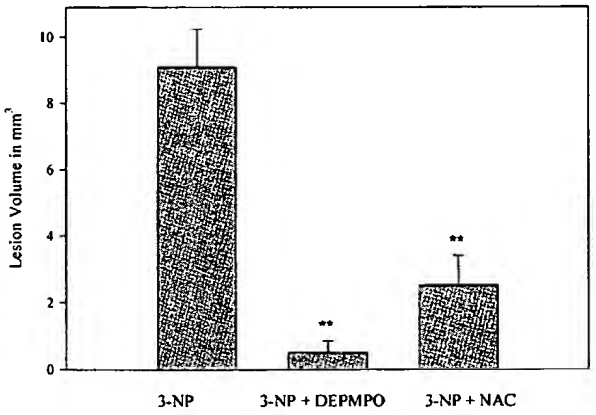

FIG. 4. Whole brains were isolated from animals treated with 3-NP, 3-NP plus DEPMPO, and 3-NP plus NAC and were sliced into 20-μm-thick cross-sections. Every 10th cross-section was stained with purple cresol (cresyl violet) tissue stain, and lesion area was measured. Lesion volume was calculated by adding the individual lesion areas and multiplying by intersectional distance. Lesion volumes in striata of animals coinjected with DEPMPO (n = 4, $p < 0.01$) and NAC (n = 4, $p < 0.01$) were significantly less than lesion volumes in striata of animals injected with 3-NP only (n = 8). $^{**}p < 0.01$.

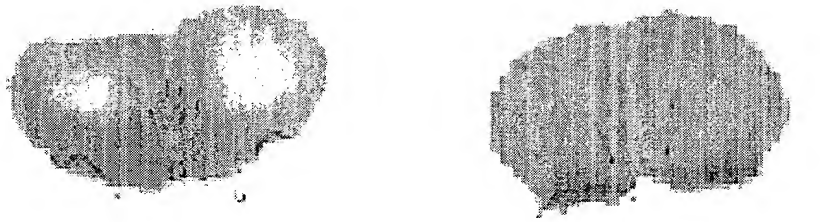

FIG. 5. Cross-section slide 400 μm into striatum of brains isolated from animals treated with 3-NP (**left**) and 3-NP + DEPMPO (**right**).

on NAC injections similar to, but not as extensive as, that seen with DEPMPO protection.

Furthermore, both DEPMPO treatment and elevated glutathione levels significantly decrease striatal lesion volume, again with DEPMPO being more effective than the NAC-induced glutathione level elevation. Because DEPMPO and glutathione both protect against oxidative damage by scavenging reactive free radicals (Fig. 6), the greater protection afforded by DEPMPO may be due to dosage. Presumably, NAC protection could be improved with greater dosage; however, because NAC itself may be toxic, higher dosage was not tested. In previous studies in this laboratory, rats were injected intraperitoneally with 300 mg of NAC/kg of body mass NAC (Pocernich et al., 2000). This dosage, however, often led to death of the animal after >2 days of injections (authors' unpublished data), so a lesser dose was chosen for this study.

The mechanisms of oxidative stress in 3-NP toxicity are as yet unknown. Possible sources of oxidation are mitochondrial impairment, NMDA receptor activation leading to an increase in superoxide production, and inflammatory response due to neuronal degeneration. Mitochondrial impairment is believed to be the major mechanism of 3-NP toxicity (Beal et al., 1993*a*). 3-NP irreversibly binds succinate dehydrogenase, an enzyme that functions to oxidize succinate to fumarate by transferring two electrons to flavin adenine dinucleotide and is the entry point of the citric acid cycle into the mitochondrial electron transport chain.

3-NP and other metabolic inhibitors also leads to removal of the Mg^{2+} block on the ion channel of NMDA receptors through depolarization of the neuronal membrane (Albin and Greenamyre, 1992; Beal, 1992). The resulting influx of Ca^{2+} may lead to increased superoxide production and activation of calcium-dependent nitric oxide synthase, causing an increase in oxidative stress (Lafon-Cazal et al., 1993; Reynolds and Hastings, 1995). There is also evidence of an inflammatory response to 3-NP toxicity, most notably increased levels of tumor necrosis factor-α (Geddes et al., 2000) and increased expression of inducible NOS (Nishino et al., 1996).

The ability of both brain-accessible spin traps and elevated levels of glutathione to attenuate 3-NP toxicity, a good model of HD, suggests antioxidants may be of therapeutic value in HD. Studies are ongoing to investi-

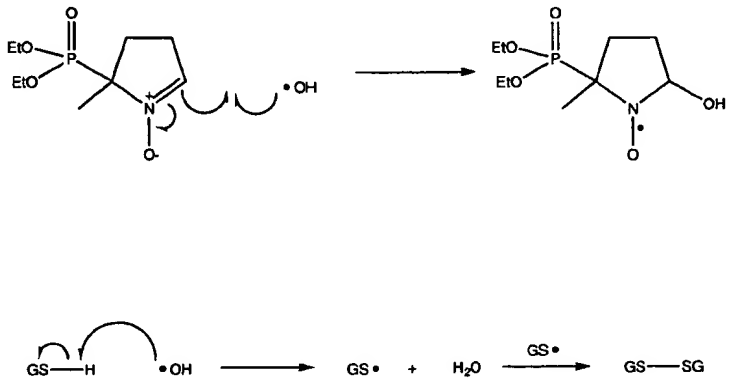

FIG. 6. Mechanism of radical scavenging of DEPMPO (**top**) and glutathione (**bottom**) with hydroxyl radical.

gate these mechanisms as a source of oxidative stress in 3-NP toxicity.

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